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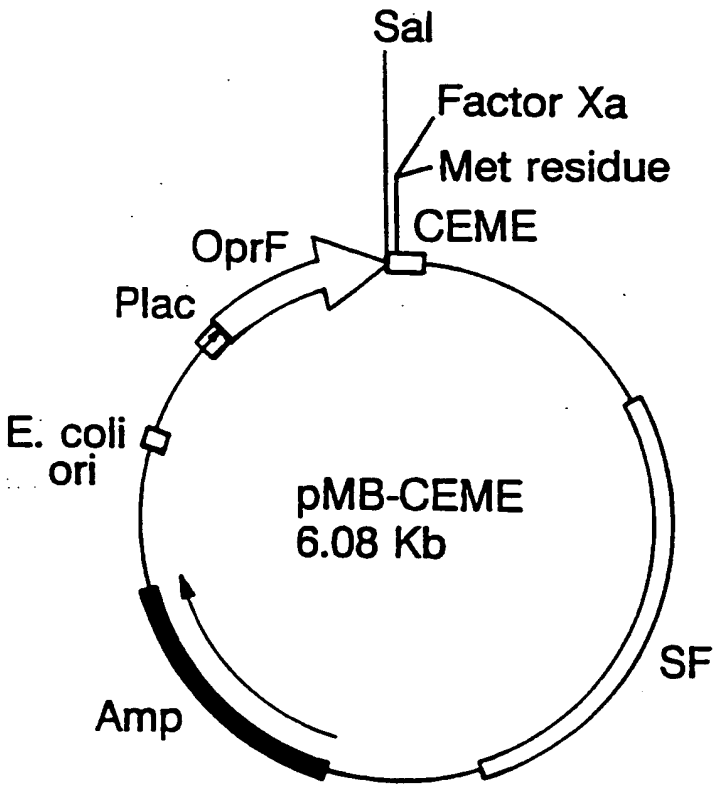
(57) Abstract

Novel compositions and methods for their preparation and use are provided comprising a coding sequence for at least the amino terminal portion of an outer membrane protein in which the one or more restriction enzyme sites have been inserted for ligation of a coding sequence for a peptide antigen, and/or to which such a peptide antigen coding sequence may be fused. The compositi ns can be synthesized or prepared by recombinant DNA technology. The compositions find use as expression systems for preparation of vaccines and as a method of identifying peptides that are useful in diagnosis of disease.



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USE OF PROTEIN OPRF FOR BACTERIAL CELL SURFACE EXPRESSION OF OLIGOPEPTIDES

INTRODUCTION

Technical Field

This invention relates to a recombinant expression system for cell surface presentation of proteins and methods for its preparation and use. The method is exemplified by use of the protein OprF as a recombinant expression system for peptide antigens.

Background

Fusion of recombinant proteins to outer membrane proteins for surface presentation or insertion into cell-surface exposed sites of integral or outer membrane proteins has been reported. Several peptides, for example, have been inserted into loops on the surface of LamB, PhoA and OmpA. Some of the limiting factors of these systems include the generally small size of acceptable inserts and that these systems have not been proven to be transferrable to gram-negative bacteria other than *E. coli*, and that the protein carriers are not known stimulators of the immune system. In addition, only a limited variety of insertion sites are available for these proteins, and epitope fusions can be performed only with a limited number of proteins using epitopes for which the corresponding DNA sequence is known.

The basic technology involves insertion of oligonucleotides, encoding peptide epitopes of interest, within the gene sequence for a surface or excreted protein such that the peptide of interest is expressed at the surface of the bacterial cell, often facing the external environment. Alternatively, one can append larger polypeptides, usually by fusing specific restriction fragments encoding these polypeptides to the amino terminal-encoding fragment of the gene for the extracellularly targeted protein. The former method, epitope insertion mutagenesis, has been performed with outer membrane proteins LamB and PhoE, and to a limited extent with OmpA and TraT. In addition, the genes for several appendages have been subjected to epitope insertion mutagenesis including those for flagellin from *E. coli* and *S. typhimurium*, and type 1, type 4, K88 and P fimbriae. Epitope fusion has been investigated to a limited extent for IgA protease, pullulanase, colicin A and OmpA.

For many of the above proteins used in epitope insertion experiments, it is not possible to utilize epitope fusion mutagenesis without interfering with synthesis and export of the protein. This is unfortunate and a great disadvantage of the LamB and PhoE systems, which have been the best developed, since this limits the versatility of the system and especially the maximal size of peptide that can be expressed, to about 60 amino acids depending on the specific insertion site in contrast to epitope fusion experiments permitting surface expression of 300 or more amino acids.

It would be of interest to develop an expression system that can be transformed to virtually any gram-negative bacteria and which may be used to express both large and small proteins with a carrier sequence that has the ability to non-specifically stimulate the immune system and itself has vaccine potential.

Relevant Literature

The expression of foreign polypeptides on the surface of *E. coli* recently has been reviewed in detail by Hofnung, *Methods in Cell Biology* (1991) 34:77-105 and most of the pertinent references in this area are included therein. Patents related to epitope insertion mutagenesis include WO8801873, EP355737, WO8910697, U.S. 4784952, EP146416 and WO8805464. References relating to the LamB and PhoE systems for epitope effusion mutagenesis include Charbit, et al., Gene (1988) 70:181-189 and Kornacker and Pugsley *Molecular Microbiology* (1990) 3:1101-1109. Applications relating to the use of OmpA for epitope insertion and epitope fusion mutagenesis include Freudl *Gene* (1990) 8:229-236 and Schorr, et al. *Vaccine* (1991) 9:675-681. A peptidoglycan associated protein (PAL) has been used to target recombinant antibodies to the surface of *E. coli*. See Fuchs, et al., Bio-Technology (1991) 9:1369-1372.

Each of the systems described in the references above involve *E. coli* proteins and genes that have not been transferred to bacteria other than *E. coli*. Knowing the sequences of these proteins and the permissive epitope insertion and fusion site do not permit one to predict the location of such sites in OprF since the protein sequences that are permissive for epitope insertion have no significant similarity to OprF (Duchene, et al. *General of Bacteriology* (1988) 170:155-162.) Further, the choice of sites in which epitopes can be inserted is limited; in some cases epitope fusion either cannot be performed or is restricted to a single site in the protein. This observation could be problematic with some antigenic peptides since it has been shown in several studies (see, for example, Van Der

Werf et al. *Vaccine* (1990) 8:269-277; Goodman-Smaitkoff et al. *Vaccine* (1990) 8:257-262; Agterberg et al. *Gene* (1990) 88:37-45; Agterburg et al. *Vaccine* (1990) 8:85-91) that the neighboring sequences of an antigenic peptide are important for maximal immunogenicity (i.e., maximal antibody production). A limitation in epitope fusion mutagenesis, in the few cases that it has been performed, has been that one must strictly maintain the sequence of triplet codons (i.e. the reading frame) so that the fused sequences are in the same reading frame. This has limited the application of this method to fusion with epitopes for which the complete DNA sequence is known and even then usually requires considerable manipulation to orient the reading frame of the DNA sequence encoding the peptide epitope with the DNA sequence encoding the outer membrane protein.

Epitopes from foot and mouth disease virus (FMDV) VP1 protein, myobacterial hsp65 (T cell epitope), poliovirus C3 epitope, hepatitis B preS2A and preS2B peptides, HIV gp120 protein, C. *trachomatis* MOMP, growth hormone releasing factor, ß-lactamase, *Plasmodium falciparum* (the malaria parasite), *Mycobacterium leprae* 65 kDa protein and cholera toxin B-subunit (e.g. Charbit et al., (1988) supra; Agterberg et al., Vaccine (1990) 8:438-440; van der Werf et al., (1990) supra have been tested. Several authors have demonstrated the ability of purified proteins with inserted epitopes to elicit a B and/or T cell response. A PhoE-FMDV hybrid was shown to protect guinea pigs against foot and mouth disease; Agterberg et al. (1990) supra, and neutralizing antibody has been elicited using several other hybrids as immunogens. Despite the large amount of data, a severe limitation on these systems has been that they have been proven useful only for continuous or linear epitopes (i.e., epitopes involving sequences of amino acids that are contiguous with the primary sequence from the antigenic protein in question). Thus, conformational epitopes involving amino acids from several parts of the sequence of the antigenic protein cannot be expressed in epitope insertion experiments, and thus largely have been ignored to date, despite the fact that such conformational epitopes are usually more prevalent than linear epitopes in antigenic proteins.

SUMMARY OF THE INVENTION

An expression system for presentation of proteins at the bacterial cell surface, together with methods for preparation and use, is provided. The expression system comprises a plasmid which includes a promoter, which may be constitutive or regulatable, and a DNA sequence encoding at least the amino terminal portion of a *P. aeruginosa* outer

membrane protein OprF. Inserted in the DNA sequence are one or more unique restriction sites for insertion of one or more DNA sequences encoding a protein(s) of interest. These inserted sequences can be known as antigenic peptides or random peptides of four or more amino acids created by utilization of randomized oligonucleotide sequences. Alternatively, or in addition, a DNA sequence encoding an oligopeptide of interest may be fused to the DNA sequence which encodes at least the amino terminal portion of the outer membrane protein. This sequence can be part of a known DNA sequence or can involve random DNA fragments from a protein of interest. The invention finds use for presentation of proteins such as peptide antigens on the cell surface of gram negative bacteria which then can be used as a live vaccine. Alternatively, or in addition, it can be used for mapping of antigenic epitopes, identifying sequences of amino acids that constitute epitopes that can be used in the diagnosis of disease, or production of specific antibodies against a given peptide sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a diagram of plasmid pRW3, the plasmid used for linker insertion mutagenesis.

Figure 2 shows the complete nucleotide sequence of pRW3.

Figure 3 shows a Western immunoblot with anti-OprF monoclonal antibody (mAb) MA7-1 against OprF produced by linker insertion mutants and native OprF.

Figure 4 shows a colony immunoblot with PF2A.10 (a monoclonal antibody specific for the PNANPNA repeating epitope of *Plasmodium falciparum* CSprotein) showing reactivity with colonies expressing an OprF derivative carrying the malarial epitope.

Figure 5 shows a Western blot of whole lysates of *E. coli* strains containing plasmids pRW302M.2 and pRW309M, respectively, with anti-OprF mAb MA7-1 (left of molecular weight marker) and anti-malarial epitope mAb PF2A.10 (right of molecular weight marker).

Figure 6 shows Western blots of whole cell proteins using either (a) an anti-OprF mAb MA7-1, or (b) a polyclonal antibody specific for CEME. Lane 1 is the wild type *P. aeruginosa* strain H103; Lane 2 is an *E. coli* strain expressing a truncated form of OprF; Lane 3 is in *E. coli* strain harboring a clone expressing wild type OprF; Lane 4 is an *E. coli* expression plasmid pMB-CEME. Symbols: square indicates band corresponding to wild type OprF; triangle indicates truncated OprF and circle indicates pMB-CEME protein, being identified with both anti-OprF mAb and anti-CEME polyclonal antibody.

Figure 7 shows examples of indirect immunofluorescent labeling experiments with anti-OprF and anti-malarial epitope mAb's. (a) *E. coli* strain expressing pRW309M with MA7-1 (anti-OprF mAb). (b) same strain with PF2A.10 (anti-malarial epitope). The upper photographs show mAb-labeled cells viewed with fluorescent filter. The bottom photographs show the same field under phase contrast.

Figure 8 shows a map of a pUC4K type plasmid. Shaded area indicates karamycin resistance cassette used for linker mutagenesis.

Figure 9 shows a diagram of plasmid pMB-CEME, an epitope fusion construct comprising the first 188 amino acids of OprF fused to a peptide construct.

BRIEF DESCRIPTION OF THE SEQUENCE LISTINGS

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

An expression system is provided which comprises a DNA sequence encoding at least the amino terminal portion of an outer membrane protein OprF, which sequence contains one or more restriction sites for insertion of a coding sequence for an oligopeptide of interest, and/or may be used for fusion to a coding sequence for an oligopeptide of interest such as a peptide antigen. The expression system also provides for DNA sequences for efficient initiation of transcription (promoter) and translation; DNA sequences for efficient termination of transcription and translation; as well as for efficient processing and transportation of the expressed protein across the outer membrane for presentation at the cell surface. The promoter is one capable of providing expression in gram negative bacteria and may be inducible or regulatable. The coding sequences for the outer membrane proteins are modifications of the coding sequence of the OprF gene from *Pseudomonas aeruginosa* PAO1. Methods for the preparation and use of the expression system also are provided.

The subject invention offers several advantages over these currently available. OprF can directly stimulate immunologically important lymphocytes and itself has vaccine potential against *Pseudomonas aeruginosa* infections (Hancock et al., European Journal of Clinical Microbiology (1985) 4:224-228; Gilleland et al., and Immunity (1988) 56:1017-1022 and recombinant OprF from *E. coli* has been used to protect against *Pseudomonas* infections (Gilleland et al., Current Microbiology (1992) 24:1-7) leading to the potential for a bipartite vaccine. OprF has been demonstrated to be a B-cell mitogen (Chen et al., Infection and Immunity (1980) 28:178-184; it also can be used as an immunogen with liposomal delivery

systems. OprF is amenable to both epitope insertion mutagenesis and epitope fusion mutagenesis. Eleven discrete sites have been isolated at which 12 nucleotides could be inserted (resulting in 4-5 amino acids being inserted into the OprF product) and at least 9 of these sites are exposed to the cell surface and are permissive for insertion of a 14 amino acid sequence containing a malaria repeating epitope PNANPNANPNA (see below). Since several sites are permissive for insertion of multiple copies of oligonucleotides encoding these peptides, a total of between about 13 and about 69 extra amino acids can be inserted. With respect to epitope fusion mutagenesis, fusions of OprF to alkaline phosphatase (at amino acid 153) and to peptides 19, 20 and 41 amino acids in length at amino acid 180, 204 and 188 respectively of OprF can be obtained. Other benefits of the system described include the expression of OprF behind a *lac* promoter to permit regulated expression; the existence of monoclonal antibodies (see, Table 2) against 10 separate epitopes of OprF (9 of which are surface exposed epitopes) which permits rapid analysis of a given product (Table 2) and the ability to express OprF in any Gram-negative bacterium, which permits live vaccine delivery.

Formulas (1) and (2) have been separately described.

Novel expression systems can include those having the following formula:

$$P\text{-}N\text{-}R_1\text{-}X\text{-}R_2\text{-}C_1 \qquad (1)$$

wherein:

P represents a DNA sequence which provides for efficient initiation of transcription in a host bacterium; the DNA sequence of OprF gene promoter must be modified to prevent overexpression lethality due to the strength of this promoter; this permits introduction of a foreign promoter which may be regulatable or constitutive. For example, the phosphate-regulatable promoter from *oprP* gene of *P. aeruginosa* or the constitutive *oprD* gene promoter.

N represents the coding sequence for the N-terminal portion of an outer membrane protein and contains a bacterial leader sequence for processing and translocation.

R_1 and/or R_2 represent restriction sites for insertion of up to about 207 nucleotides encoding an oligopeptide of interest; the number of restriction sites is about 1 to 4 at R_1 or R_2;

X represents the central portion of the outer membrane protein.

C represents the C terminal portion of the outer membrane protein.

Novel expression systems also include compositions which have the following formulas:

$$P\text{-}N_1\text{-}R_1 \quad (2)$$

$$P\text{-}N_1\text{-}R_1\text{-}C_1 \quad (3)$$

wherein:

N_1 represents the coding sequence for the N-terminus of outer membranae protein OprF and is characterized as providing for expression of a sufficient amount of a consecutive sequence of amino acids from the N-terminus (about 153 or more amino acids) to permit expressions of a peptide fused at R_1 to be expressed on the surface of the outer membrane protein, as well as providing the coding sequence for a bacterial leader sequence to permit processing and translocation to the outer membrane;

C_1 represents either an actual OprF carboxy terminus or a synthetic carboxy terminus having substantially the sequence of a native OprF carboxy terminus;

P and R_1 have the meaning as described above under Formula (1).

A variety of outer membrane proteins are of interest. In particular, although the actual gene and protein have not been identified, nearly all bacterial species rRNA homology group 1 of the Family *Pseudomonadaceae*, and certain related species, possess either a sequence that cross-hybridizes with the OprF gene (Ullstrom, et al. Journal Bacteriol (1991) 173:768-775) or a protein that cross-reacts with OprF-specific monoclonal antibodies (N. Martin, Ph.D. Thesis (1992) University of British Columbia, Canada), unlike the proteins from *E. coli* that have been used previously for epitope insertion or epitope fusion mutagenesis. It is expected that these proteins would have similar characteristics to OprF, namely ability to accept additional peptides at multiple sites, and predictable sites based on the information presented here; activity as a B-cell mitogen; ability to function in several bacterial species; and ability to be used for both epitope insertion and epitope fusion. Proteins that are capable of exhibiting such characteristics, including those mentioned above, are of interest in the subject invention as a source of nucleic acid sequences capable of providing for expression of an oligopeptide of interest on the surface of an outer membrane protein.

The compositions can be prepared by taking DNA encoding at least the N-terminal portion of an outer membrane protein and creating one or more restriction enzyme sites within the coding sequence where a DNA sequence encoding a heterologous protein can be inserted so as to obtain a fusion protein in which an oligopeptide of interest is inserted

into the outer membrane protein sequence. The altered gene can be expressed in a host prokaryotic cell, particularly a bacterial cell, more particularly a gram negative bacterial cell.

The techniques used in isolating outer membrane protein genes to obtain the desired sequences are known in the art, including synthesis, isolation from genomic DNA, or combinations thereof. Various techniques for manipulation of genes are well known, and include restriction, digestion, resection, ligation, *in vitro* mutagenesis, primer repair, employing linkers and adapters, and the like (see Sambrook et al., Molecular Cloning, a Laboratory Manual, Cold Spring Harbor, USA, 1989). Generally, the method comprises preparing a genomic library from an organism expressing an outer membrane protein with the desired characteristics. The genome of the donor microorganism is isolated and cleaved by an appropriate restriction enzyme, such as *Eco* R_1. The fragments obtained are joined to a vector molecule which has previously been cleaved by a compatible restriction enzyme. An example of a suitable vector is plasmid PLAFR3 which can be cleaved by the restriction endonuclease *Eco* R_1.

The amino acid sequence of an outer membrane protein also can be used to design a probe to screen a cDNA or a genomic library prepared from mRNA or DNA from cells of interest as donor cells for an outer membrane protein gene. By using the outer membrane protein cDNA or a fragment thereof as a hybridization probe, structurally related genes found in other microorganisms can be easily cloned. The probes can be considerably shorter than the entire sequence but should be at least 18, preferably at least 21, nucleotides in length. Longer oligonucleotides are also useful, up to the full length of the gene, preferably no more than 500, more preferably no more than 250, nucleotides in length. RNA or DNA probes can be used.

In use, the probes are typically labeled in a detectable manner for example with ^{32}P, ^{3}H, biotin or avidin) and are incubated with single-stranded DNA or RNA from the organism in which a gene is being sought. Hybridization is detected by means of the label after single-stranded and double-stranded (hybridized) DNA (or DNA/RNA) have been separated (typically using nitrocellulose paper). Hybridization techniques suitable for use with oligonucleotides are well known to those skilled in the art. Although probes are normally used with a detectable label that allows easy identification, unlabeled oligonucleotides are also useful, both as precursors of labeled probes and for use in methods that provide for direct detection of double-stranded DNA (or DNA/RNA). Accordingly, the term "oligonucleotide probe" refers to both labeled and unlabeled forms. Particularly

contemplated is the isolation of genes from organisms that express outer membrane proteins using oligonucleotide probes based on the nucleotide sequences of the OprF gene obtainable from *Pseudomonas aeruginosa*.

Once a nucleotide sequence encoding an outer membrane protein has been identified, as a restriction fragment of chromosomal DNA, it can then be manipulated in a variety of ways to prepare an expression system which has a structure represented by formula (1) above. The constructs comprising the expression system may include functions other than those required for expression, such as replication systems in one or more hosts, e.g. cloning hosts and/or the target host for expression of the protein of interest; one or more markers for selection in one or more hosts, as indicated above; genes which enhance transformation efficiency; or other specialized functions.

The construct may be prepared in conventional ways, by isolating genes of interest from an appropriate host, by synthesizing all or a portion of the genes, or combinations thereof. Similarly, the regulatory signals, the transcriptional and translational initiation and termination regions may be isolated from a natural source, be synthesized, or combinations thereof. The various fragments may be subjected to endonuclease digestion (restriction), ligation, sequencing, in vitro mutagenesis, primer repair, or the like. The various manipulations are well known in the literature and will be employed to achieve specific purposes.

The various fragments may be combined, cloned, isolated and sequenced in accordance with conventional ways. After each manipulation, the DNA fragment or combination of fragments may be inserted into the cloning vector, the vector transformed into a cloning host, e.g. *E. coli*, the cloning host grown up, lysed, the plasmid isolated and the fragment analyzed by restriction analysis, sequencing, combinations thereof, or the like. Various vectors may be employed during the course of development of the construct and transformation of the host cell. These vectors may include cloning vectors, expression vectors, and vectors providing for integration into the host or the use of bare DNA for transformation and integration.

The cloning vector will be characterized, for the most part, by a marker for selection of a host containing the cloning vector and optionally a transformation stimulating sequence, may have one or more polylinkers, or additional sequences for insertion, selection, manipulation, ease of sequencing, excision, or the like.

Expression of an oligopeptide of interest is achieved by insertion of one or more open reading frame(s) encoding an oligopeptide of interest(s) into a restriction site created in the sequence encoding the outer membrane protein. Insertions are made by using a variety of molecular genetic techniques which are well known in the art. The structure of the modified genes can vary significantly depending on the location of the restriction enzyme site into which the sequence encoding the antigen of interest is inserted. For example, see Formulas (1) to (3) above. By analogy to other expression systems it might be necessary to construct different insertion and/or fusion genes and to determine experimentally the optimal fusion construct for expression. Alternatively, or in addition, a bank of oligonucleotides varying in length from about 12 to 24 nucleotides and including every possible nucleotide (ACG or T) at every position along the length of the oligonucleotide can be inserted into the restriction enzyme site of choice; resulting, after transformation of cells with these constructs, in a series of derivatives of every possible combination of amino acid sequence in peptide inserts varying from 4 to 8 amino acids in length to create a Variable Epitope Library.

It is desirable to locate the protein of interest in a region of the outer membrane protein that is exposed on the surface of the protein. Such regions may be identified by inserting a known epitope (e.g. PNANPNANPNA) for which a monoclonal antibody is available, and examining intact cells producing the outer membrane protein with this known epitope by indirect immunofluorescent techniques using the monoclonal antibody. Positive fluorescence will reveal a surface-localized epitope and consequently a region exposed to the surface that is a permissive site for insertion of epitopes. Generally, these regions correspond to a loop region that falls between two transmembrane β-sheets although such regions are notoriously difficult to predict in outer membranae proteins. Once these regions are identified, they can then be mutated to create unique restriction enzyme sites, and an oligonucleotide may be used as need to create a unique site. It is advantageous to create at least two unique restriction sites per plasmid within the outer membrane protein gene to permit the construction of proteins expressing two or more separate peptide epitopes. It is desirable to maintain the signal sequence (secretory leader) of the outer membrane protein upstream from and in reading frame with the outer membrane coding sequence gene. As few as about 7 amino acids from the N-terminus are required so long as the C-terminus is present.

Alternatively, the nucleotide sequence encoding the outer membrane protein may be ligated to a DNA sequence encoding an oligopeptide of interest. For such an epitope fusion construct, it is advantageous to include the signal sequence and at least about 150 to 180 amino acids from the N-terminus of the outer membrane protein. This involves placement by mutagenesis of restriction sites at or after the position in the DNA sequence equivalent to amino acid 150 (See Table 1). Inclusion of all of the nucleotides encoding three blunt ended restriction endonuclease sites placed such that the corresponding restriction endonucleases cut each in a different reading frame (Table 3, pAS2) results in an expression system that can be utilized to randomly clone DNA fragment generated either by use of similar restriction endonucleases that cut in a blunt-ended fashion, or by deoxyribonuclease I digestion in the presence of Mn^{2+} or by sonication to create a Fragment Library for a given protein for which the corresponding gene sequence does not have to be known. The resulting ligated DNA will usually express an antigenic epitope or can then be manipulated in a variety of ways to provide for expression.

Expression vectors will usually provide for insertion of a construct which includes the transcriptional and translational initiation region and termination regions; alternatively the construct may lack one or both of the regulatory regions, which will be provided by the expression vector upon insertion of the sequence encoding the protein product.

Illustrative transcriptional regulatory regions or promoters include, the lambda left and right promoters, *trp* and *lac* promoters, *tac* promoter, and the like. The transcriptional regulatory region may additionally include regulatory sequences which allow the time of expression of the fused gene to be modulated, for example the presence or absence of nutrients or expression products in the growth medium, temperature, etc. How to obtain and use a promoter to obtain a particular level or timing of expression is well known to these skilled in the art. (See for example, Deuschle et al. EMBO Journal (1986) 5:2987-2994; Soldat et al FEMS Microbiology Letters (1987) 42:163-167.

The expression cassette can be included within a replication system for episomal maintenance in an appropriate cellular host or can be provided without a replication system, where it can become integrated into the host genome. The DNA can be introduced into the host in accordance with known techniques.

Microbial hosts can be employed which can include, for example gram negative bacteria from the Family *Enterobacteriaceae* such as *E. coli* and those from the

family *Pseudomonadaceae* such as *Pseudomonas aeruginosa*. Although the outer membrane protein expression system may have been obtained from a particular bacterial host, it has been determined for OprF that the system can be used with other gram negative bacterial hosts. Heterologous expression of promoters, terminators and secretion signals is a common observation in studies on gene expression in gram negative bacteria. Since outer membrane proteins are highly expressed in their native state, it is desirable to delete their normal promoter to prevent overexpression lethality in high copy number plasmids, and to permit a choice of promoters to be used for expression of the outer membrane protein.

Virtually any peptide sequence can be inserted into the outer membrane protein by means of the insertion of synthetic oligonucleotides or natural DNA sequences at specific permissive sites in the outer membrane protein gene. Such permissive sites can be inserted into the protein and identified as described above. In epitope insertion experiments, i.e. corresponding to Formula (1), a maximum size limit of 69 amino acids (207 nucleotides) has been observed (Table, pRW311M), although up to 100 amino acids may be tolerated. The amino acids inserted can be the known sequences of linear (continuous) peptide epitopes that comprise the dominant antigenic portion of organisms including but not limited to viruses, fungi and other bacteria, or can include a Variable Epitope Library as described above. Antibodies against a specific organism or peptide sequence can then be utilized to ensure that the antigen in question is expressed in the former case. Alternatively, antisera can be used to select reactive clones from the Variable Epitope Library of clones, and the DNA sequences corresponding to the inserted epitopes in these permissive clones can be determined. This permits identification of the unknown epitope sequences corresponding to antibody reactivities in the antisera.

For epitope fusion experiments (i.e. corresponding to Formulas (2) and (3), up to 400 amino acids can be fused at or after amino acid 153 of the mature outer membrane protein by fusion of DNA sequences to the region of the outer membrane protein corresponding to amino acid 153 or greater. The DNA sequence to be fused can be fully synthetic, or can be a portion of a gene of interest (for example, an antigenic protein from an organism, which antigenic protein is known to give rise to antibodies that protect against infections) or if no such gene is known, random DNA sequences from the chromosome of a bacteria of interest. These latter two possibilities are termed a "Fragment Library" above and clones containing sequences corresponding to antigens of interest can be identified using specific antibodies.

Conditions are employed for transformation which result in a high frequency of transformation, using either natural or induced transformation systems or by electroporation so as to ensure selection and isolation of transformed hosts expressing the structural gene(s) of interest. It will be appreciated that the transformed host according to the invention can be used as starting strain in strain improvement processes other than DNA mediated transformation. The resulting strains are considered to form part of the invention.

As a result of the transformation, there will be at least one copy of the gene(s) of interest frequently two or more, usually not exceeding about 100, more usually not exceeding about 10. The number will depend upon whether integration or stable episomal maintenance is employed, the number of copies integrated, whether the subject constructs are subjected to amplification and the like.

Once the altered gene including insertions and/or fusions has been introduced into the appropriate host, the host can be grown to express the altered gene. Where a regulatable promoter such as a *lac* promoter is used, expression of the mutated outer membrane protein is controlled by the amount of regulator nutrient in the growth medium. The insertion and/or fusion constructs are transformed into a gram negative bacterium host, preferably *P. aeruginosa* or *E. coli* or live vaccine strains of *Salmonella* or *Francisella*, by methods known in the art. Transformants are selected by using a bacterial selection marker such as tetracycline resistance. The structural gene providing the marker for selection or maintenance of the plasmid may be native to the wild-type bacterial host or a heterologous structural gene which is functional in the host. For example, structural genes coding for an enzyme in a metabolic pathway may be used where the structural gene is functional in the host and complements the auxotrophy to prototrophy.

Transformants are purified and tested for expression of the antigen of interest. This is done using specific antibodies in a colony immunoblot test after transfer of these colonies to nitrocellulose paper. When Variable Epitope Libraries or Fragment Libraries are being screened, specific clones of interest will be enriched for by using their ability to bind to specific antibodies bound to either a bead support in columns or to the surface of plastic dishes. Bound clones will be those which express an antigenic sequence corresponding to the antibodies. These clones can be eluted by mild acid or high salt, grown up and subjected to further cycles of enrichment prior to testing by colony immunoblot methods.

In a preferred embodiment the DNA sequences of the expression system can be derived from an OprF gene, which encodes outer membrane protein, isolated from the

gram negative bacterium *P. aeruginosa* in which organism (as indeed it is in *E. coli*) it is expressed and efficiently translocated to the outer membrane. The cloned outer membrane protein of *P. aeruginosa* can also be used to create mutant outer membrane protein genes with improved expression and/or secretion characteristics by using molecular genetic techniques well known in the art. It is also recognized that hybrid sequences for expression and secretion of proteins can be obtained by combining outer membrane protein secretion signal sequences with other promoter or terminator sequences. Outer membrane protein gene promoter, secretion signal and optionally terminator sequences, or functional parts thereof, can be obtained and used as individual cassettes in complete expression systems.

Moreover, the invention includes genes with different nucleotide sequences which are homologies of the outer membrane protein gene of *P. aeruginosa* or parts thereof. Homology is defined herein as nucleotide sequences which have an identity score of at least 70% in a sequence comparison to outer membrane protein by using the BestFit program of the Wisconsin Sequence Analysis Software Package (version 6.0, release 1989, GCG, University of Wisconsin, USA), using parameter settings gap penalty = 4, bias parameter = 0. Homolog genes may be isolated from natural sources, or may be produced by mutagenesis of outer membrane protein genes of *P. aeruginosa*.

The subject invention exemplifies a method to efficiently express peptide antigens in a gram negative bacterium such as *P. aeruginosa*, and *E. coli* using regulatory sequences obtainable from an outer membrane protein. The invention provides conservative mutations, where the sequence may have as many as 30% different bases, more usually not more than about 10% different bases, or mutations which are non-conservative.

The isolation of the outer membrane protein gene allows use of the regulatory elements of the outer membrane protein gene, such as a promoter, an upstream activating sequence (UAS), a terminator and the like, for identification of other specific regulatory sequences by means of standard techniques such as gel retardation, cross-linking, DNA footprinting and the like. Isolation of specific regulatory protein by affinity chromatography will result in the cloning of the gene encoding said protein and subsequent manipulation in a suitable host.

Use of Expression Systems

The uses of these expression systems include the potential for expressing antigenic peptides of interest in live vaccine strains of bacteria. In such a situation, the antigenic peptide would comprise a sequence that could give rise to an immune response

leading to antibodies or activated T cells capable of protecting against subsequent infection by a pathogenic organism which includes this antigenic peptide on its surface. The advantage of this invention for such purposes include (a) the ability to express the antigenic peptide at the surface of the live vaccine strain by incorporation into the outer surface of the outer membrane protein, which itself is expressed on the surface of the bacterial cell, (b) the known vaccine potential of OprF against *P. aeruginosa* infections, thus creating the potential for bipartite vaccine, (c) the potential for fusion of large antigenic portions of proteins permitting the expression of conformational epitopes (i.e. discontinuous epitopes), (d) the potential for expression of two peptide epitopes within the same construct due to the presence of two or more unique restriction sites with the embodiments of this expression system, (e) the activity of the protein as a B-cell mitogen leading to non-specific priming of antibody-producing B-lymphocytes and (f) the ability to express OprF in heterologous bacteria.

A second use is to prepare a protein vaccine by purification using standard detergent solubilization and column chromatography techniques of the protein expressing an antigenic peptide, as described above. OprF, being capable of being inserted into liposomes (Hancock et al., European Journal of Clinical Microbiology (1985) 4:224-228) could be delivered in such a formulation as a vaccine. For such use, all of the advantages (a) to (e) above would be apparent.

A third use is to prepare antibodies against a given peptide sequence. Thus, the peptide could be inserted into the outer membrane protein gene as a complementary oligonucleotide. The protein product is then purified and used for immunization of animals. Antibodies so raised that are specific for OprF sequences can be absorbed out using OprF bound to an affinity column matrix. The resultant antibodies are peptide specific. Again, points (a) to (e) represent obvious advantages in this system. Another advantage of OprF is that since two of the insertion sites exist within a region of OprF that forms one or two disulfide bridges between cysteine residues, the insert at these sites of antigenic peptides that must form disulfide loops for antigenicity, becomes a potential usage of this system.

A fourth use of this system is for the identification of important antigenic protein sequences for use as diagnostics or vaccines. This involves creation of a Variable Epitope Library or Fragment Library followed by selection of relevant clones using an anti-sera that is capable of identifying (i.e. diagnosing) all strains of a given species of organism, or an antisera that can protect against infection by the organism. Sequencing of the relevant clones will reveal antigenic epitopes of interest. These can then be used directly (i.e. by use

of the original OprF with the inserted peptide) or indirectly (i.e. by using this information to synthesize peptides) for diagnostic tests or as vaccines.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

OprF plasmid preparation

The approach utilized to isolate the plasmid pRW3 for creation of OprF epitope insertion vectors was as follows. The OprF gene promoter was mutated by site directed mutagenesis to place a unique HindIII site overlapping the -10 site of the promoter. This had three effects. First, it weakened the promoter. This was important to permit subcloning into high copy number vectors since OprF is such a highly expressed protein (10^5 copies per cell) in both *P. aeruginosa* and *E. coli*. Second, it permitted subcloning behind regulated promoters. The promoter-mutated gene was subcloned into plasmids pTZ19R for expression in *E. coli* or PUCP19 for expression in *P. aeruginosa*. Third, it resulted in a form of the OprF gene that completely lacked a *Pst*I site and had a single unique *Sal*I site. Also, it removed most of the sequences flanking the OprF, thus increasing the efficiency of linker insertion mutagenesis.

Linker mutagenesis of plasmid containing OprF gene

The plasmid pRW3 (Figure 1), which contains the whole OprF gene with a mutated promoter in pTZ19R, was linearized with 4 different restriction enzymes (AluI, HaeIII, RsaI and ThaI) which leave blunt ends after digestion. Since all 4 enzymes recognized more than 1 site in the plasmid, different partial digestion conditions were set up for each enzyme in order to obtain the singly cut linearized form of pRW3. After partial digestions, the reaction mixtures were loaded on preoperative agarose gel and the linear form of the plasmid was isolated using DEAE paper. The 4 pools of linearized pRW3, each corresponding to a different restriction enzyme digestion, were ligated separately with a 1.3 kb HincII fragment which encoded a kanamycin resistance (aminoglycoside 3'-phosphotransferase) gene derived from a pUC4K type plasmid (Figure 8). Following ligations and transformations, cells were plated on Luria agar plates containing 50 mg/ml each of kanamycin and ampicillin. The doubly resistant colonies were further screened on colony immunoblots for an OprF-minus phenotype by using an anti-OprF monoclonal

antibody. The loss of OprF phenotype indicated the insertion of the kanamycin resistance cassette in the OprF gene sequence.

Plasmid DNA from the OprF-minus colonies was extracted by the alkaline lysis method. The extracted plasmid DNA from each OprF-minus clone was then digested with *Pst*1, which only recognized sites in the flanking sequence of the kanamycin resistance cassette (Figure 8), and hence cleaved the cassette out from the plasmid. Following relegation of the *Pst*1 digestion mixtures and transformations, cells were plated in ampicillin medium. Colonies that appeared were screened for kanamycin sensitivity and recovery of production of immunoreactive OprF. The kanamycin sensitive clones presumably contained the mutated forms of pRW3 with a 12 nucleotide insertion at sites originally interrupted by the kanamycin resistance cassette.

Plasmid DNA was prepared from the kanamycin sensitive clones and the insertion sites were mapped by restriction pattern analysis by double digestion with *Pst*1, which recognized the unique site generated by the 12 nucleotide linker, and other enzymes with single cleavage sites in the oprF sequence. Clones with the same restriction pattern were grouped and 1 clone from each group was further analyzed by automated DNA sequencing using dyeterminator chemistry. For clone 307, a slightly different technique was used. The unique *Sal*1 restriction site centered around the sequence encoding amino acid 188 was opened with *Sal*1 and a linker sequence with *Sal*1 overlapping ends and 12 extra nucleotides containing a *Pst*1 site (thus replacing valine 188 with the sequence, gly-pro-ala-gly-pro) was inserted into this site. In all, 11 unique insertion sites were identified. The 12 nucleotide insertions were translated to 4 amino acids, the identities of which depended on the reading frame at which the insertions occurred (Table 1).

Table 1: Summary of linker insertion mutants.

Clones	Insertion Sites	Amino acids inserted	Non-reactive anti-OprF MAbs	Malaria epitope inserted	Number of a.a's inserted
pRW301	Gly-2	GTCRS	none	yes	39
pRW302	Ala-26	GPAGP	none	yes	14,28
pRW303	Asp-42	DLQV	all	yes	14
pRW305	Gly-131	GPAGP	7-1	yes	28
pRW306	Gly-135	GTCRS	none	yes	26
pRW307	Val-188	GPAGP	7-8	yes	42
pRW308	Gly-196	GPAGP	7-8, 4-4	yes	14
pRW309	Arg-211	RTCRS	none	yes	39
pRW310	Asp-215	DLQV	none	no	–
pRW311	Ser-231	RTCRS	7-3, 7-4, 7-5, 7-7	yes	69
pRW312	Arg-290	RTCRS	7-3, 7-4, 7-5, 7-7	yes	65

Characterizations of OprF produced by the linker insertion mutants

Outer membrane samples were prepared from *E. coli* strains containing different mutated forms of pRW3. Samples were electrophoresed on SDS polyacrylamide gel and analyzed by Western blots using a series of 10 monoclonal antibodies specific for native OprF (Figure 3). Certain of the mutated forms of OprF showed different reactivity patterns with these monoclonal antibodies as compared to the native protein, indicating that certain epitopes were interrupted in these mutated proteins (Table 1). However, reactivity of the mutated proteins with the majority of the monoclonal antibodies indicated substantial retention of native OprF structure.

Insertion of oligonucleotides encoding the malaria epitope into linker insertion mutants

Synthetic oligonucleotides encoding the malaria circumsporozoite (CS) protein repeating sequence PNANPNANPNA were inserted into nine of the mutated forms of pRW3

at the unique *PstI* site generated by the 12 nucleotide linker (Table 1). The recombinants were screened on colony immunoblots with 2 different monoclonal antibodies specific for the inserted sequence (Figure 4) as well as with OprF-specific monoclonal antibodies to demonstrate retention of OprF (Table 2). Plasmid DNAs from the positive clones were extracted and digested with *SphI*, which recognized a unique site generated by the epitope-specifying sequence. The cleavage of the plasmid DNA by *SphI* thus further confirmed the presence of the malarial sequence in the mutated pRW3 derivative plasmids.

Table 2: Monoclonal antibodies that can be used to characterize fusion and insertion products.

Monoclonal antibodies	Epitopes recognized (Amino acid positions)	Surface localization
MA7-1	24 - 112	yes
MA7-2	198 - 275	no
MA7-3	188 - 245	yes
MA7-4	188 - 275	yes
MA7-5	188 - 275	yes
MA7-6	198 - 240	yes
MA7-7	188 - 275	yes
MA7-8	176 - 187	yes
MA4-4	176 - 187	yes
MA5-8	300 - 320	yes

Surface exposure of the malarial epitope

The surface exposure of the malarial epitope was detected by indirect immunofluorescent labelling. Cells expressing the recombinant OprF with inserted malaria epitopes (Table 3) were incubated with a 100 fold dilution of monoclonal antibody specific for the malarial epitope. After washing with PBS, cells were incubated with a 20 fold dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The treated cells were examined under a Zeiss microscope fitted with a condenser for fluorescence microscopy and containing a halogen lamp and suitable filters for emission of fluorescein isothiocyanate at 525nm (Figure 7). The retention for surface-localized OprF epitopes was demonstrated in similar indirect immunofluorescent labelling using OprF specific monoclonal antibodies.

Use In Epitope Fusion Experiments

Preliminary experiments demonstrated that as a result of insertion of transposon Tn*PhoA* into the *oprF* gene, stable gene fusions could be isolated which produced protein fusions of alkaline phosphatase (a 47,000 molecular weight, 436 amino acid protein) attached to the first 153 amino acids of OprF or peptides of 19 and 20 amino acids attached to the first 180 or 204 amino acids respectively of OprF. Therefore, a 135 based pair oligonucleotide was synthesized and inserted into the unique *Sal*1 site (at amino acid 188) of pRW3. This oligonucleotide (Table 3, pMB-CEME) encoded an in-frame factor X protease cleavage site followed by a methionine followed by the sequence for a 26 amino acid bacteriocidal protein construct called CEME (a hybrid of insect cecropin and be venom melitin) and translational stop sites in all three reading frames (i.e. creating a new construct according to formulas (2) and (3)). Expression of this construct (Figure 9) (containing 41 new C-terminal amino acids fused to OprF) was demonstrated by immunoblotting of whole cell proteins using both a monoclonal antibody specific for the amino terminus of OprF (MA7-1) and a polyclonal antibody specific for CEME (Figure 6). The factor X cleavage site and the methionine permit potential relief of the peptide by enzymatic or chemical means. Subsequently, the sequence shown in Table 3 (plasmids PAS1 and PAS2) was inserted into the *Sal*1 site of the *oprF* gene. This permits blunt-end ligation in all three reading frames of the *oprF* gene (each using a separate restriction enzyme) to ensure, in one of the three cases, the alignment of the reading frame of any DNA fragment cloned into the sites. The source of these DNA fragments could include genes of interest randomly cut with deoxyribonuclease 1 in the presence of Mn^{2+} or by sonication (or when no genes of interest are known, randomly cut chromosomal DNA).

Table 3: The inserted sequences and insertion sites of all clones derived from pRW3, including the fusion protein construct pMB-CEME. Position 1 is the OprF transcription start site.

Clones	Inserted Sequences[1]	Nucleotide positions of insertions
pRW301	GACCTGCAGGTC	135
pRW302	GACCTGCAGGTC	206
pRW303	GACCTGCAGGTC	256
pRW305	GACCTGCAGGTC	521
pRW306	GACCTGCAGGTC	534
pRW307	GACCTGCAGGTC	694
pRW308	GACCTGCAGGTC	716
pRW309	GACCTGCAGGTC	768
pRW310	GACCTGCAGGTC	775
pRW311	GACCTGCAGGTC	822
pRW312	GACCTGCAGGTC	997
pRW301M	GACCTGCA $(ME2F)_3$ GGTC	135
pRW302M.1	GACCTGCA (ME1F) GGTC	206
pRW302M.2	GACCTGCA $(ME1F)_2$ GGTC	206
pRW303M	GACCTGCA (ME1B) GGTC	256
pRW305M	GACCTGCA (ME1F, ME1B) GGTC	521
pRW306M	GACCTGCA (ME2F, ME2B) GGTC	534
pRW307M	GACCTGCA (ME1B, ME1F, ME1F) GGTC	694
pRW308M	GACCTGCA (ME1F) GGTC	716
pRW309M	GACCTGCA $(ME1F)_3$ GGTC	768
pRW311M	GACCTGCA $(ME2F)_{5\frac{1}{2}}$ GGTC	822
pRW312M	GACCTGCA $(ME2F)_5$ GGTC	997

continued

Table 3 (Continued)

Clones	Inserted Sequences[1]	Nucleotide positions of insertions
pAS1	GACCTGCAGGCCTGTCGCGATATCC TGCAGGTC	206
pAS2	GACCTGCAGGATATCGCGACAGGC CTGCAGGTC	206
pMB-CEME	CGTCGACATCGAAGGTCGTGCATGC GGGGATCCGCATATGAAATGGAAA CTGTTCAAGAAGATCGGCATCGGCG CCGTGAAAGTGCTGACCACCGGTCT GCCGGCGCTCAGCTAACTAAGTAAG CTTGTCGAC	694

[1] Sequences inserted are:
ME1FCCGAACGCCAACCCGAACGCCAACCCACGCCCGGGCAT GCA; ME1B-the reverse of ME1F;
ME2FACCCGAACGCCAACCCGAACGCCAACCCGAACGCATGC A; ME2B-the reverse of ME2F.
ME1F corresponds to the malarial epitope sequence in the GPAGP reading frame (Table 1) in the correct orientation.
ME1B corresponds to the malarial epitope sequence in the GPAGP reading frame (Table 1) in the reverse orientation.
ME2F corresponds to the malarial epitope sequence in the TCRS reading frame (Table 1) in the correct orientation.
ME2B corresponds to the malarial epitope sequence in the TCRS reading frame (Table 1) in the reverse orientation.

The invention described here is a process by which antigenic regions (epitopes), which are found in a pathogenic organism and which can be utilized as a vaccine for raising protective antibodies in humans, can be expressed on the surface of an outer membrane protein OprF. The main component of this invention is a series of 11 plasmids (Fig. 1, Table 1) each containing an engineered cloned oprF gene for the *Pseudomonas aeruginosa* major outer membrane protein OprF into which has been inserted a 12 nucleotide linker region at a different site of the OprF gene for each of the 11 plasmids (Table 1). Insertion of the 4 extra amino acids encoded by these linkers still permit production of OprF in *E. coli* (Fig. 2) and inclusion of a unique *Pst*I restriction site within the linker region permits either the insertion of synthetic oligonucleotides encoding specific epitopes (Fig. 3, Table 1) or the potential fusion of larger epitopes to the OprF amino terminal-encoding portion of the gene prior to the *Pst*I site. Inclusion of a malarial epitope (PNANPNANPNA)

at all 9 of the sites tested (Table 1), and its expression on the surface of *E. coli* containing the 9 plasmids with inserted epitopes in such a form that it reacts with 2 malaria-specific monoclonal antibodies, demonstrates the potential of this system. In addition, all plasmids except plasmid 307 have an additional unique *Sal*I site. Results with insertions into this *Sal*I site (in plasmid 307; Table 1) show that this site can also be utilized for insertion of epitopes giving 10 of the plasmids the potential for simultaneous insertion of 2 epitopes. Furthermore this *Sal*I site has been demonstrated to be capable of acting as a receptor site for fusion of a DNA sequence encoding at least 41 amino acids to the region coding for the first 188 amino acids of OprF. The data further show that OprF can be expressed in both *P. aeruginosa* and *E. coli*, giving this system great potential in live vaccine therapy.

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A vaccine comprising an effective amount of bacterial cells consisting essentially of cells expressing on their surface one or more antigens heterologous to said cells.

2. The vaccine according to Claim 1, wherein said vaccine is a live vaccine.

3. The vaccine according to Claim 1, wherein said bacterial cells are gram negative bacterial cells.

4. The vaccine according to Claim 3, wherein said gram negative bacterial cells are selected from the group consisting of *Escherichia coli* and *Pseudomonas aeruginosa*.

5. The vaccine according to Claim 1, wherein said antigen is obtainable from a disease causing organism.

6. The vaccine according to Claim 5, wherein said disease causing organism is malaria parasite.

7. A method of immunizing a mammal comprising:

the step of administering an effective amount of bacterial cells consisting essentially of cells expressing on their surface one or more antigens heterologous to said cells.

8. A method of producing an improved vaccine comprising:

the step of growing a culture of bacterial cells consisting essentially of cells transformed to express on their surface one or more antigens heterologous to said cells.

9. A DNA sequence encoding an amino acid sequence represented by a formula selected from the group consisting of:

(1) P-N-R_1-X-R_2-C_1, (2) P-N_1-R_1, and (3) P-N_1-R_1-C_1

wherein in (1), P represents a DNA sequence which provides for efficient initiation of transcription in a host bacterium, N represents the coding sequence for the N-terminal portion of an outer membrane protein and contains a bacterial leader sequence for processing

and translocation, R_1 and R_2 represent restriction sites for insertion of up to about 207 nucleotides encoding an oligopeptide f interest and the number of restriction sites is about 1 to 4 at R_1 or R_2, X represents the central portion of the outer membrane protein, and C represents the C terminal portion of the outer membrane protein; and

wherein in (2) and (3), N_1 represents the coding sequence for the N-terminus of outer membrane protein OprF and is characterized as providing for expression of a sufficient amount of a consecutive sequence of amino acids from the N-terminus to permit expression of a peptide fused at R_1 to be expressed on the surface of the outer membrane protein, as well as providing the coding sequence for a bacterial leader sequence to permit processing and translocation to the outer membrane, C_1 represents either an actual OprF carboxy terminus or a synthetic carboxy terminus having substantially the sequence of a native OprF carboxy terminus, P and R_1 have the meaning as described above under (1).

10. The DNA sequence according to Claim 9, wherein said bacterial outer membrane protein is selected from the group consisting of OprF and homologs of OprF.

11. A plasmid comprising the DNA sequence according to Claim 9 under control of a promoter functional in gram negative bacteria.

12. The plasmid according to Claim 11, wherein said promoter is a regulatable promoter.

13. The plasmid according to Claim 12, wherein an epitope of interest is inserted in one or more of said linker regions.

14. The plasmid according to Claim 13, wherein said epitope of interest is a malarial epitope.

15. The plasmid according to Claim 14, wherein said malarial epitope is PNANPNANPNA.

16. Gram negative bacteria which are transformed with a plasmid according to 11.

17. Gram negative bacteria according to Claim 16, wherein said bacteria are selected from the group consisting of *Escherichia coli* and *Pseudomonas aeruginosa*.

18. The plasmid according to Claim 12, wherein a protein or peptide of interest is inserted in one of said linker regions to provide a fusion protein comprising an N-terminus of at least 153 amino acids of the outer membrane protein fused to a C-terminus comprising said protein or peptide of interest.

19. The plasmid according to Claim 18, wherein said protein or peptide of interest is a construct comprising a factor X-cleavage site fused to a methionine residue fused to cecropin-melitin hybrid sequence.

20. The plasmid according to Claim 18, wherein said protein or peptide of interest has an amino acid sequence substantially as follows:
IEGRACGDPHMKWKLFKKIGIGAVLKVLTTGLPALIS.

21. A method for producing a mutant gram negative bacterial strain expressing a heterologous antigenic sequence on the cell surface, said method comprising the steps of:

transforming a gram negative bacterial strain with a plasmid comprising a DNA sequence according to Claim 9 into which sequence one or more heterologous nucleotide sequences encoding antigenic sequence(s) have been inserted to obtain a transformed cells;

selecting by means of a marker gene in said plasmid said transformed cells;

isolating cells which express said antigenic sequence(s) on their surface from among said transformed cells, whereby a mutant strain is obtained.

22. A *Pseudomonas aeruginosa* strain prepared according to the method of Claim 18.

23. Plasmid pRW3.

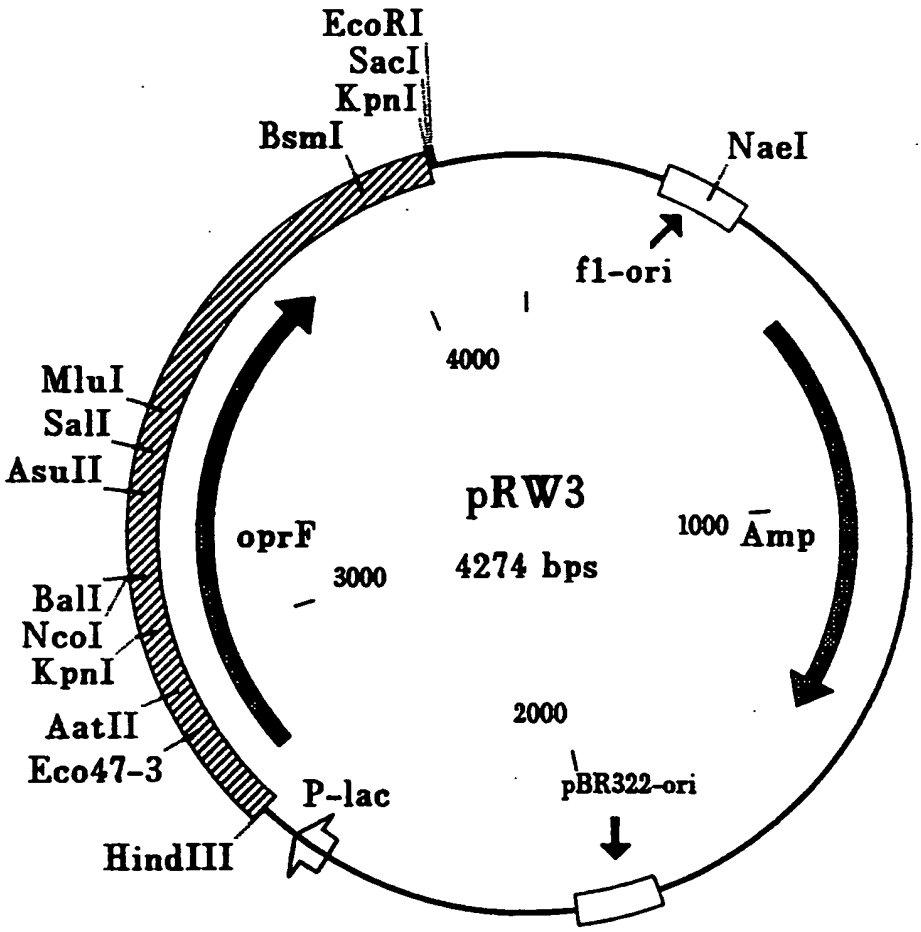
EcoRI
SacI
KpnI
BsmI
NaeI
f1-ori
4000
MluI
SalI
AsuII
pRW3
4274 bps
oprF
3000
1000
Amp
BalI
NcoI
KpnI
AatII
Eco47-3
2000
pBR322-ori
P-lac
HindIII

FIG. 1

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AAATTGTAAACGTTAATATTTTGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAA 112

TCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAAAAACCGTCTATCAGGGCG 224

ATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCACTAAATCGGAACCCTAAAGGGATGCCCCGATTTAGAGCTTGACGGGGAAA 336

GCCGGCGAACGTGGCGAGAAAGGAAGGGAAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGCTTAAT 448

GCGCCGCTACAGGGCGCGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAAC 560

CCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCC 672

AGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAA 784

CGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACT 896

TGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCT 1008

GACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGAC 1120

GAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGG 1232

ATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCC 1344

AGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG 1456

TAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCC 1568
```

FIG. 2A

CTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACC 1680

ACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCG 1792

TAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGG 1904

ACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCG 2016

TGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCC 2128

TGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTT 2240

TACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCG 2352

CAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGAC 2464

AGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTG 2576

GAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTTAATACGACTCACTATAGGGAAAGCTTGCCACCCAAGTTGTGCGGCTGATTGTT 2688

GGACAACTAACTGACCATCAAGATGGGGATTTAACGGATGAAACTGAAGAACACCTTAGGCGTTGTCATCGGCTCGCTGGTTGCCGCTTCGGCAATGAACGCCTTTGCCCAG 2800

GGCCAGAACTCGGTAGAGATCGAAGCCTTCGGCAAGCGCTACTTCACCGACAGCGTTCGCAACATGAAGAACGCGGACCTGTACGGCGGCTCGATCGGTTACTTCCTGACCG 2912

ACGACGTCGAGCTGGCGCTGTCCTACGGTGAGTACCATGACGTTCGTGGCACCTACGAAACCGGCAACAAGAAGGTCCACGGCAACCTGACCTCCCTGGACGCCATCTACCA 3024

CTTCGGTACCCCGGGCGTAGGTCTGCGTCCGTACGTGTCGGCTGGTCTGGCTCACCAGAACATCACCAACATCAACAGCGACAGCCAAGGCCGTCAGCAGATGACCATGGCC 3136

FIG. 2B

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AACATCGGCGCTGGTCTGAAGTACTACTTCACCGAGAACTTCTTCGCCAAGGCCAGCCTCGACGGCCAGTACGGTCTGGAGAAGCGTGACAACGGTCACCAGGGCGAGTGGA 3248

TGGCTGGCCTGGGCGTCGGCTTCAACTTCGGTGGTTCGAAAGCCGCTCCGGCTCCGGAACCGGTTGCCGACGTTTGCTCCGACTCCGACAACGACGGCGTTTGCGACAACGT 3360

CGACAAGTGCCCGGATACCCCGGCCAACGTCACCGTTGACGCCAACGGCTGCCCGGCTGTCGCCGAAGTCGTACGCGTACAGCTGGACGTGAAGTTCGACTTCGACAAGTCC 3472

AAGGTCAAAGAGAACAGCTACGCTGACATCAAGAACCTGGCTGACTTCATGAAGCAGTACCCGTCCACTTCCACCACCGTTGAAGGTCACACCGACTCCGTCGGCACCGACG 3584

CTTACAACCAGAAGCTGTCCGAGCGTCGTGCCAACGCCGTTCGTGACGTACTGGTCAACGAGTACGGTGTAGAAGGTGGTCGCGTGAACGCTGTTGGTTACGGCGAGTCCCG 3696

CCCGGTTGCCGACAACGCCACCGCTGAAGGCCGCGCTATCAACCGTCGCGTTGAAGCCGAAGTAGAAGCCGAAGCCAAGTAATCGGCTGAGCCTCTAAGGAAAAACCCGGCC 3808

CAGGCCGGGTTTTTCTTTGCCTGGAAAAAGACCGCTCGTCAGGCGCTCAGGGAAATCGGTTGCGACACGATGTCGCGGGTTACTTCGCCGATCACCAGGATCGCCGGGCTTT 3920

TCAAGGCGAAACGTCCGGCGTCCCGCAGTAGCTCGCCGAGGTTGCTGCGGCATTCGCGCTGGTTGCCCAGGGTGGCGTTCTCGATCATCGCCAGCGGCGTATCCTCGGCCAT 4032

ACCTCCAGCCAGCAGCCCGGCCTGGATCTCTGCGAGACGGGCGACGCCCATGTAGACCACCAGCGTGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGAC 4144

TGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCA 4256

GCCTGAATGGCGAATGGG                                                                                                  4274
```

FIG. 2C

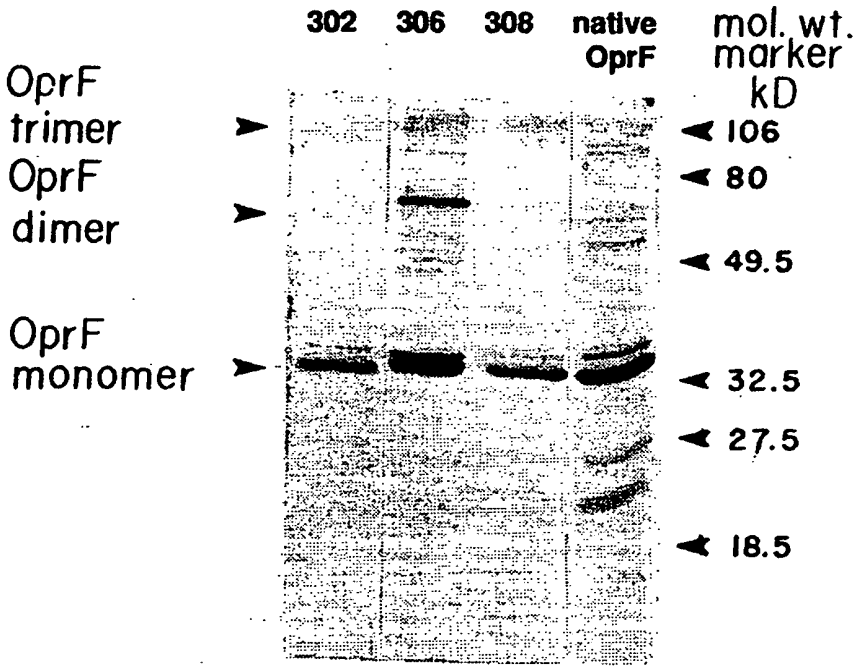

302
306
308
native OprF
mol. wt. marker kD
OprF trimer
OprF dimer
OprF monomer
106
80
49.5
32.5
27.5
18.5

FIG. 3

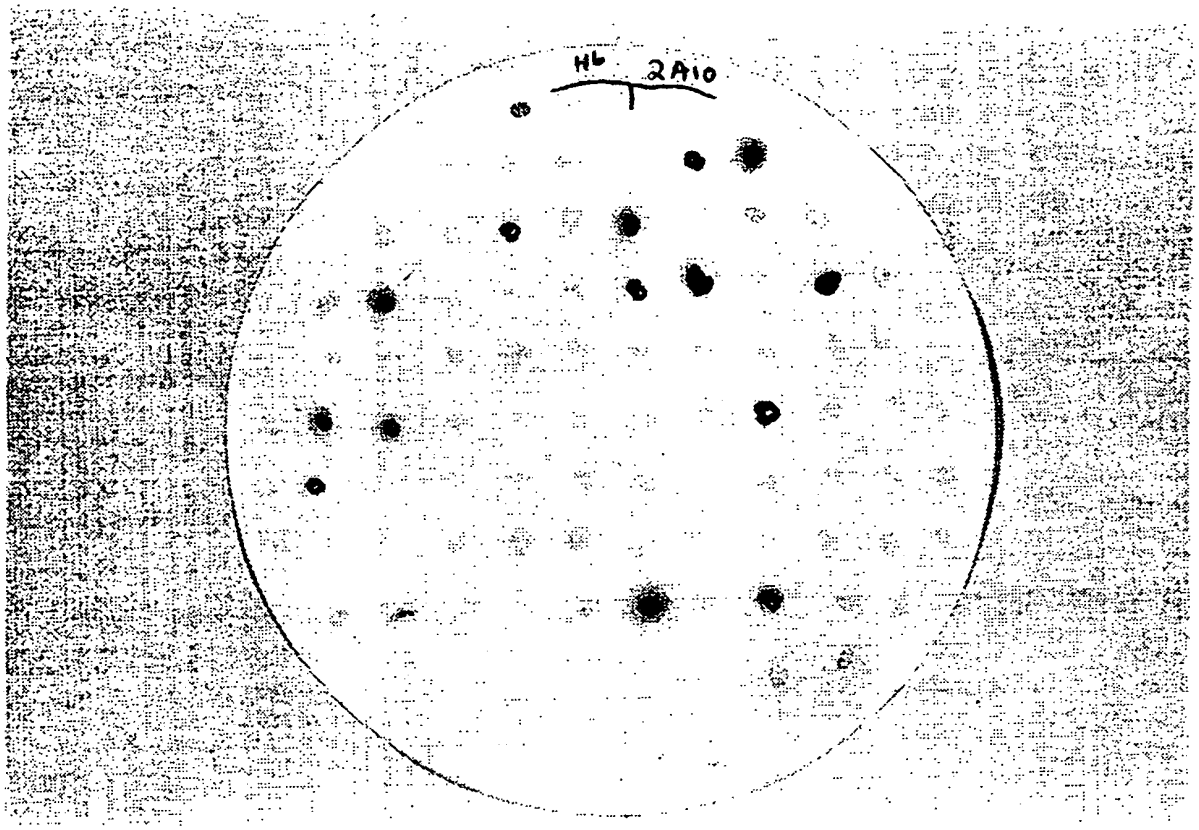


FIG. 4

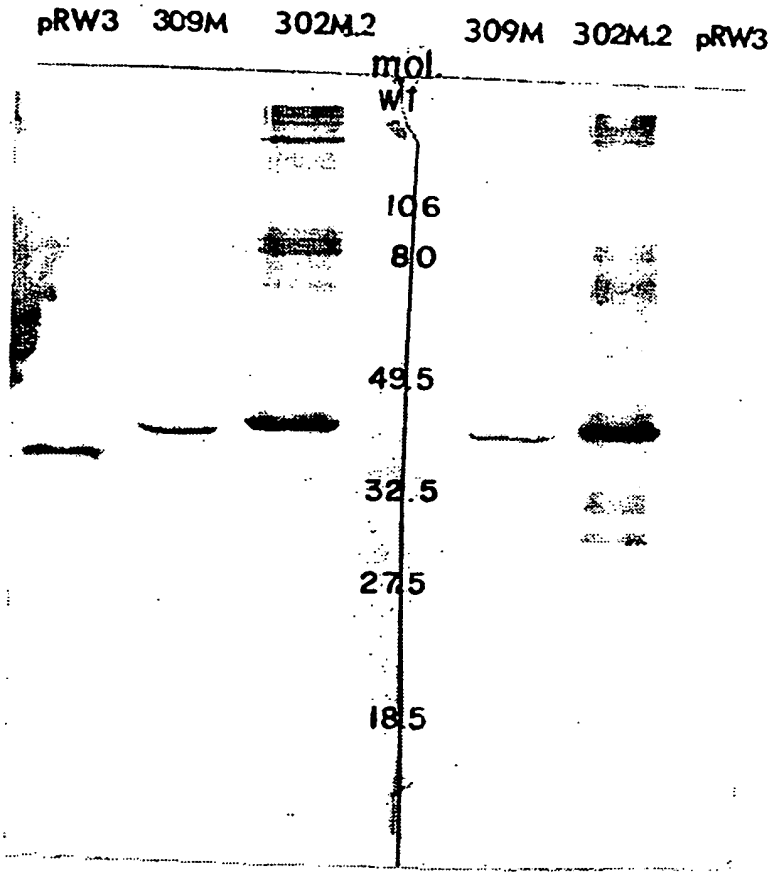


FIG. 5

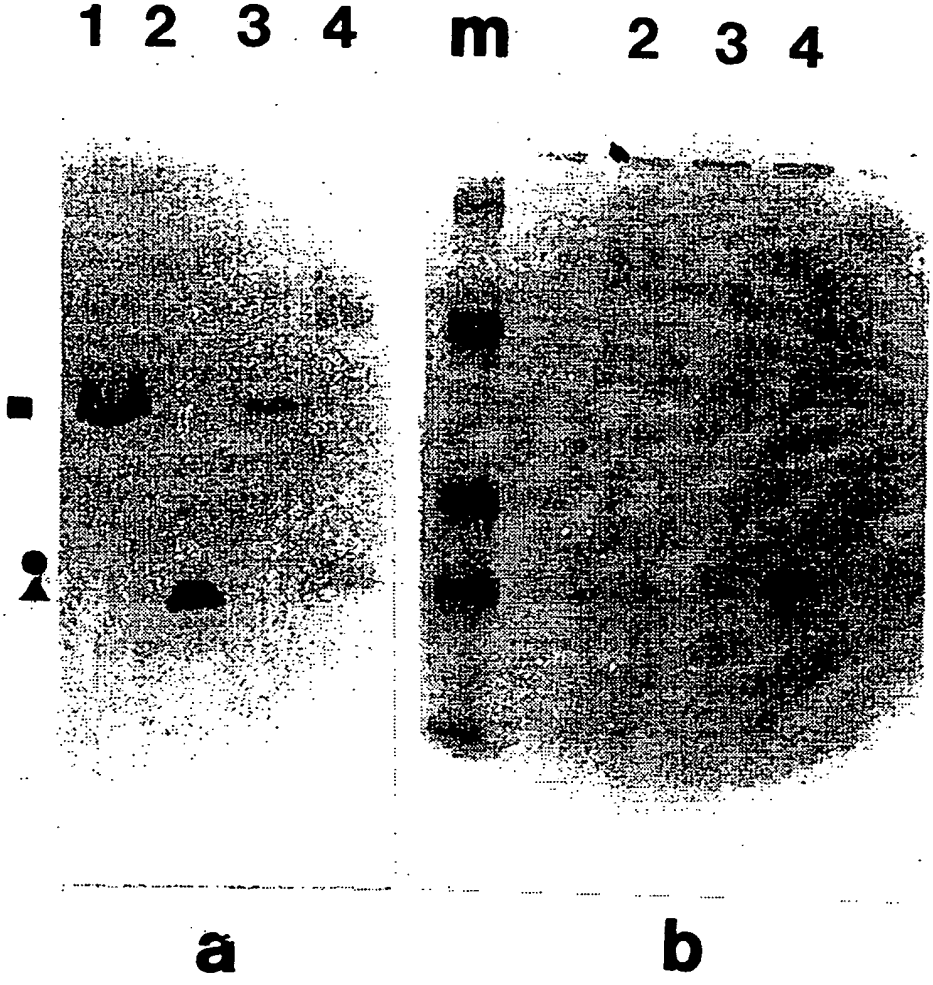


FIG. 6

FIG. 7a

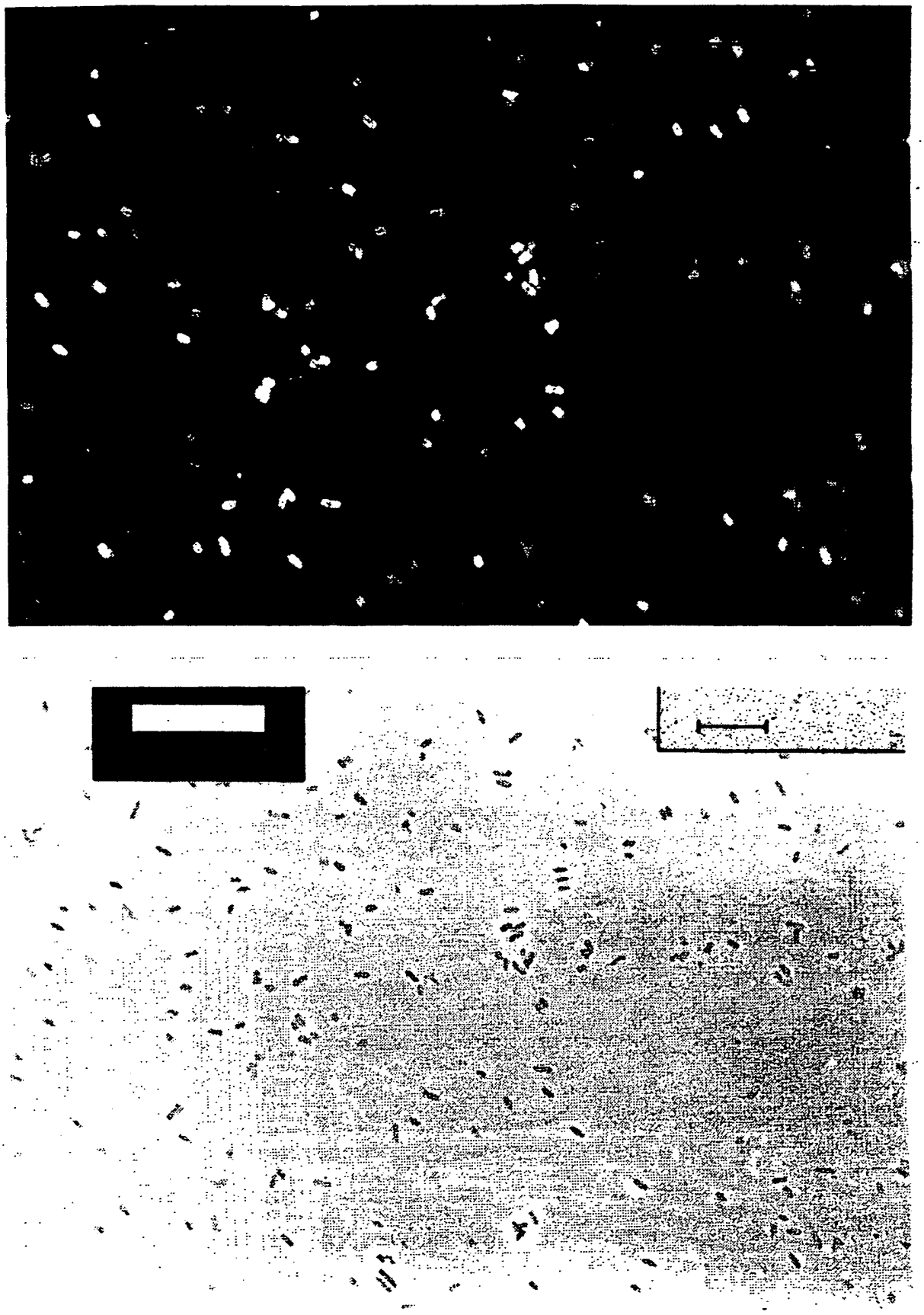
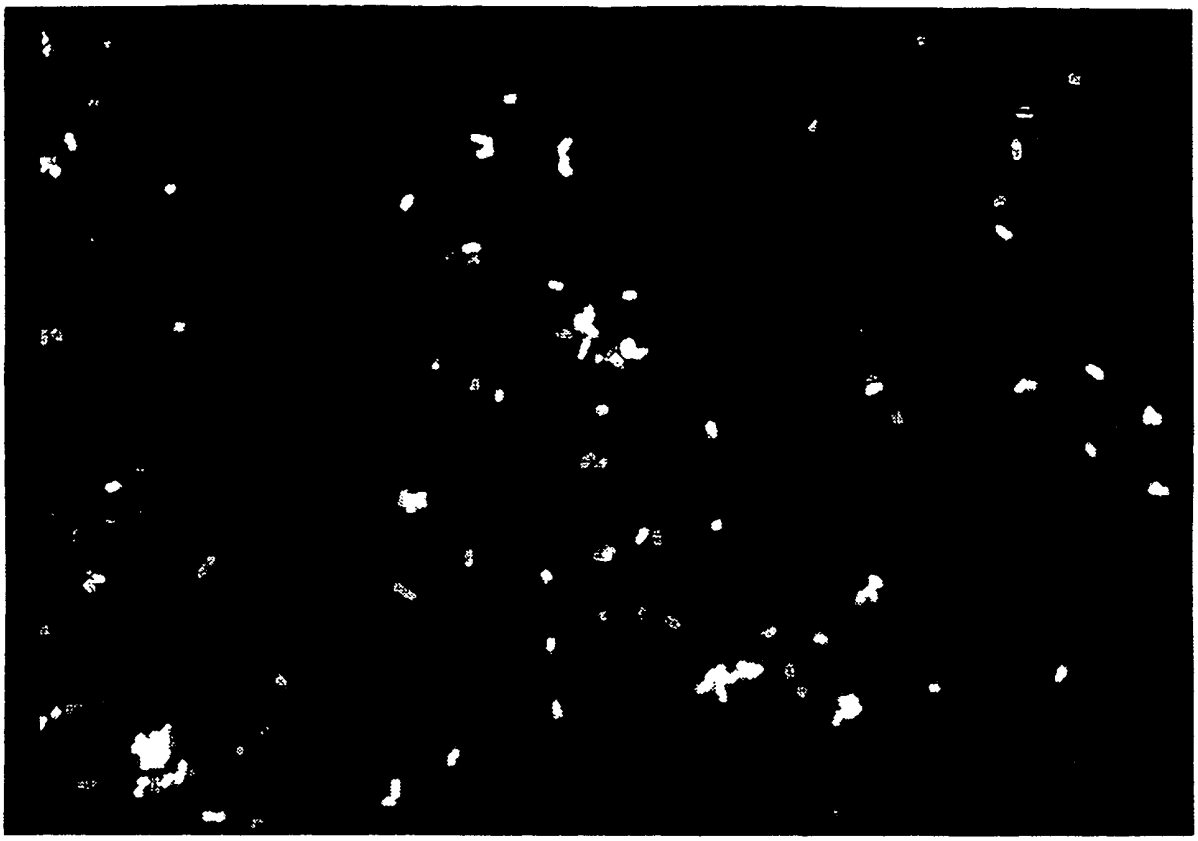
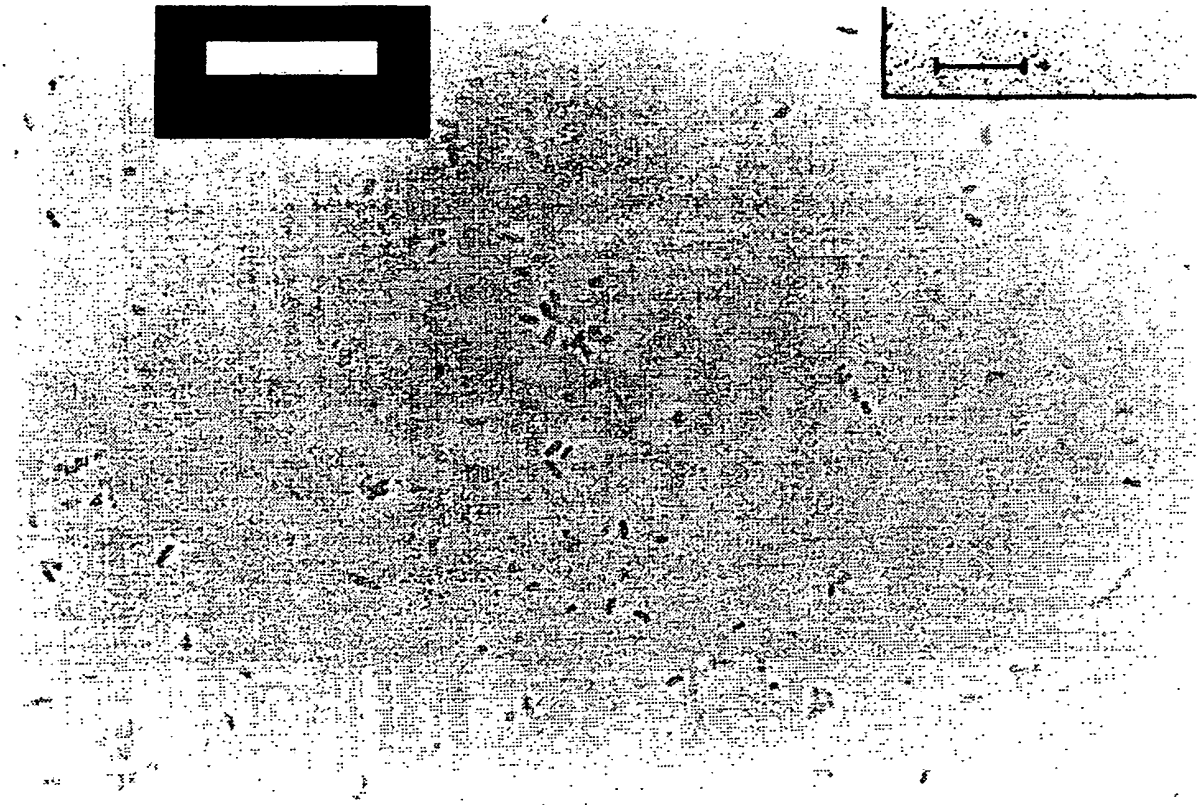


FIG. 7b





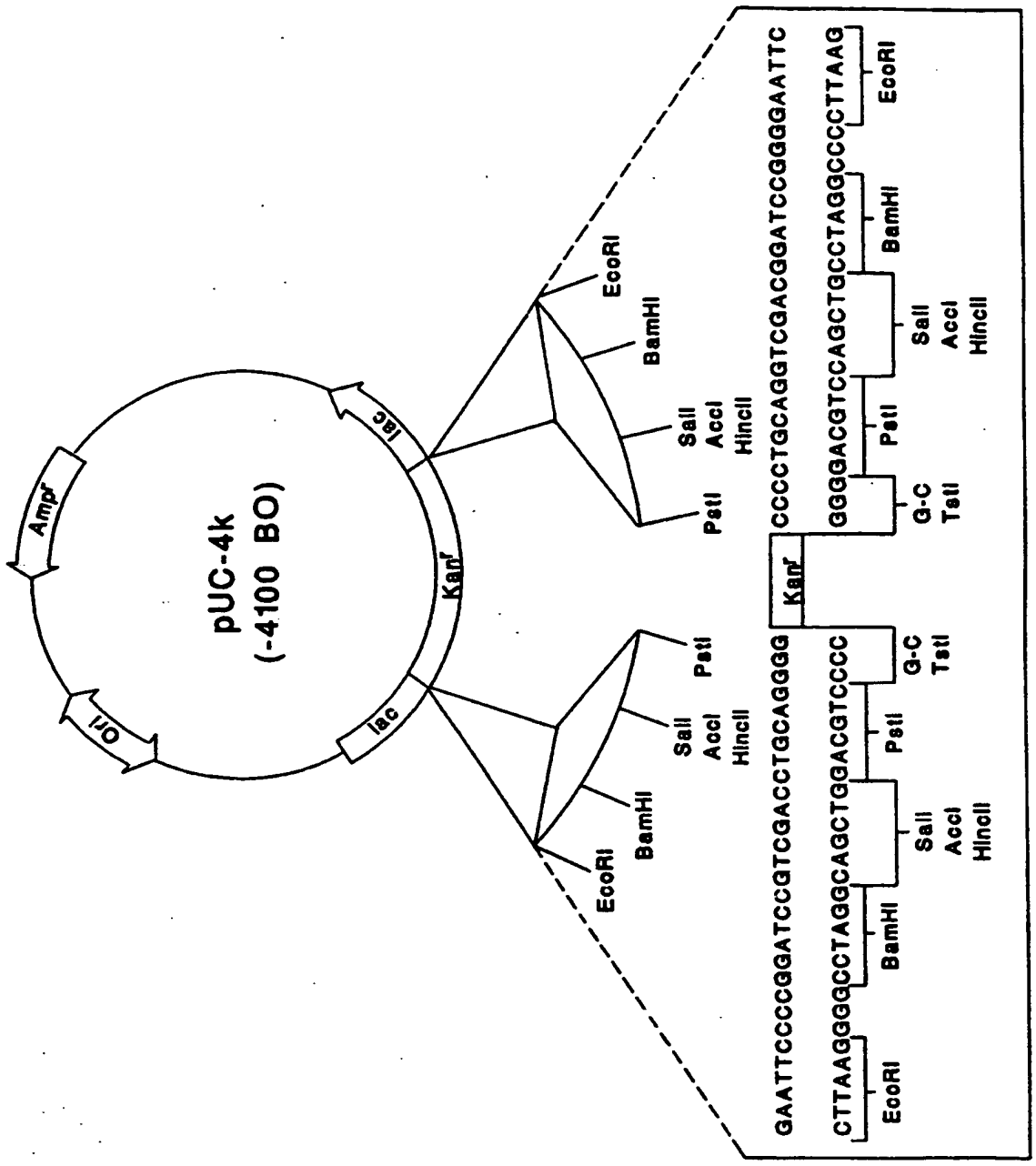

pUC-4k
(-4100 BO)
Ampr
ori
lac
Kanr
lac
Kanr
EcoRI
BamHI
SalI
AccI
HincII
PstI
GAATTCCCCGGATCCGTCGACCTGCAGGGG
CTTAAGGGGCCTAGGCAGCTGGACGTCCCC
CCCCTGCAGGTCGACGGATCCGGGGAATTC
GGGGACGTCCAGCTGCCTAGGCCCCTTAAG
G-C
TstI

FIG. 8

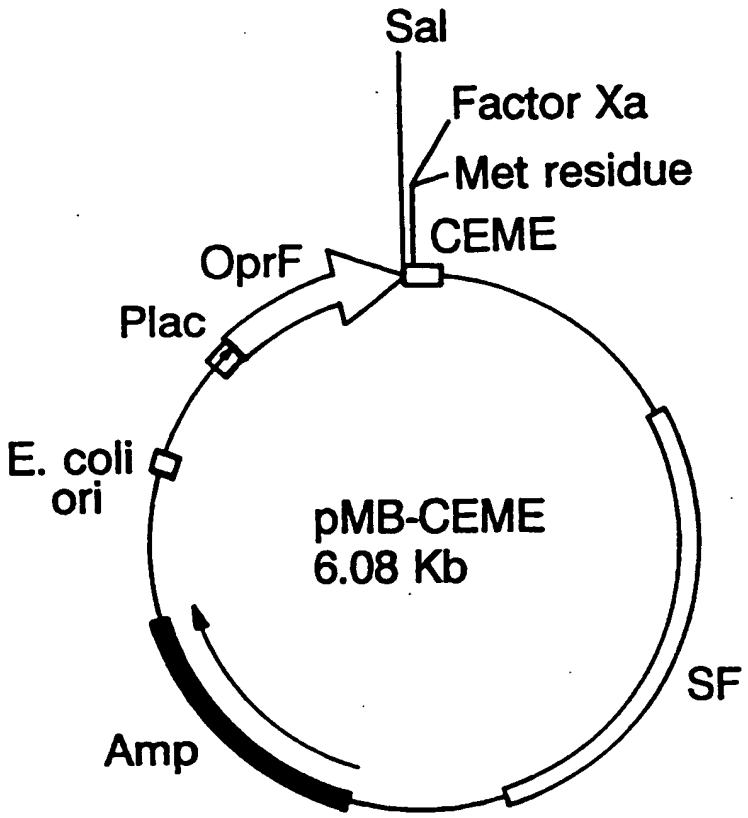

Sal
Factor Xa
Met residue
CEME
OprF
Plac
E. coli
ori
pMB-CEME
6.08 Kb
SF
Amp

FIG. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 93/00227

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/62; C12N15/31; A61K39/015; C12N1/21
// C12N15/12,(C12N1/21,C12R1:385,1:19)

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,8 805 464 (RIJKSUNIVERSITEIT UTRECHT & STICHTING CENTRAAL DIERGENEESKUNDIG INSTITUT) 28 July 1988 cited in the application see page 6, line 13 - page 8, line 33	1-5,7-9, 11-13, 16-18,21
Y	see page 11, line 24 - page 12, line 10	6,10,14

-/--

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07 SEPTEMBER 1993	28 -09- 1993
International Searching Authority	**Signature of Authorized Officer**
EUR PEAN PATENT OFFICE	ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	CURRENT MICROBIOLOGY vol. 24, no. 1, January 1992, NEW YORK, USA pages 1 - 7 GILLELAND, H. ET AL. 'Recombinant outer membrane protein F of Pseudomonas aeruginosa elicits antibodies that mediate opsonophagocytic killing, but not complement-mediated bacteriolysis, of various strains of P.aeruginosa' cited in the application see the whole document	10
Y	BIOTECHNOLOGY vol. 6, no. 9, September 1988, NEW YORK US pages 1065 - 1070 RUTGERS, T. ET AL. 'Hepatitis B surface antigen as carrier matrix for the repetitive epitope of the circumsporozoite protein of Plasmodium falciparum' see the whole document	6,14
X	EP,A,0 146 416 (INSTITUT PASTEUR & INSERM & CNRS) 26 June 1985 cited in the application see page 3, line 12 - page 4, line 4 see claims	1-5,7-8
X	EP,A,0 355 737 (BEHRINGWERKE A.G.) 28 February 1990 cited in the application see the whole document	1-5,7-8
X	METHODS IN CELL BIOLOGY vol. 34, 1991, pages 77 - 105 HOFNUNG, M. 'Expression of foreign polypeptides at the Escherichia coli cell surface' cited in the application see page 82, paragraph B see page 94, paragraph B see page 99, paragraph A	1-5,7-8
A	WO,A,9 011 771 (THE ROCKEFELLER UNIVERSITY) 18 October 1990 see the whole document	19

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 297 291 (BEHRINGWERKE A.G.) 4 January 1989 see the whole document	

INTERNATIONAL SEARCH REPORT

I. .national application No.

PCT/CA93/00227

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 7 is directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

CA 9300227
SA 74663

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 07/09/93
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-8805464	28-07-88	NL-A-	8700127	16-08-88
EP-A-0146416	26-06-85	FR-A-	2551456	08-03-85
		JP-A-	60149388	06-08-85
EP-A-0355737	28-02-90	DE-A-	3828666	01-03-90
		AU-B-	614174	22-08-91
		AU-A-	4016089	01-03-90
		JP-A-	2135095	23-05-90
WO-A-9011771	18-10-90	CA-A-	2031199	13-10-90
		EP-A-	0422215	17-04-91
		JP-T-	3501742	18-04-91
EP-A-0297291	04-01-89	DE-A-	3718591	15-12-88
		AU-B-	617318	28-11-91
		AU-A-	1697788	08-12-88
		JP-A-	1085086	30-03-89